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Covalent-Allosteric Kinase Inhibitors**

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Abstract: *Targeting and stabilizing distinct kinase conformations is an instrumental strategy for dissecting conformation-dependent signaling of protein kinases. Herein the structure-based design, synthesis, and evaluation of pleckstrin homology (PH) domain-dependent covalent-allosteric inhibitors (CAIs) of the kinase Akt is reported. These inhibitors bind covalently to a distinct cysteine of the kinase and thereby stabilize the inactive kinase conformation. These modulators exhibit high potency and selectivity, and represent an innovative approach for chemical biology and medicinal chemistry research.*

Over the last two decades, kinases have evolved as central targets for drug discovery in academic and industrial research.[1] Dysregulated kinases in diseases such as cancer, autoimmune disorders, neurodegenerative diseases, and diabetes have been addressed by a vast diversity of small-molecule inhibitors.[2] Despite the highly conserved ATP-binding site in kinases throughout the human kinome, a number of orthosteric inhibitors has been identified and approved by the FDA, including examples such as Erlotinib and Gefitinib.[3] Furthermore, allosteric kinase inhibitors that stabilize enzymatically inactive conformations[4] and covalent inhibitors that irreversibly target rare non-catalytic cysteines have recently been shown to be beneficial for increased selectivity and potency.[5] These characteristics translate into high efficacy and reduced off-target reactivity, which offer significant benefits for targeted cancer therapies.[6]

The protein kinase Akt features a regulatory pleckstrin homology (PH) domain, along with the catalytic kinase domain, allowing for membrane attachment upon growth factor stimulation and activation by phosphorylation by the upstream kinase PDK1.[7] PI3K/Akt signaling mediates most of the cellular processes which comprise the hallmarks of cancer.[8] Therefore, Akt dysregulation is directly associated with neoplastic transformation and malignant progression as well as increased resistance to chemo- and radiotherapy in a variety of solid tumors, such as breast, prostate, and colorectal cancer.[9] To this end, chemical modulation of dysregulated Akt using selective small molecule inhibitors provides a promising perspective for the treatment of patients suffering from diverse forms of cancer.[8] Allosteric PH domain-dependent Akt inhibitors, such as the clinical candidate MK-2206,[10] exhibit an exclusive binding mode. They target a unique pocket at the interface of the regulatory PH and the catalytically active kinase domain, thereby stabilizing the inactive "PH-in" conformation (Figure 1).[11] Recently, kinase-independent but conformation-dependent functions of Akt were identified that promote cancer cell survival.[12] Chemically modulating and interfering with such inter-domain interactions is a major opportunity for understanding kinase function beyond catalysis. X-ray structure analysis of full-length Akt in complex with allosteric inhibitors Akti-1/2 (PDB code: 3O96)[13] and **12j** (PDB code: 4EJN)[14] revealed the presence of non-catalytic A-loop cysteines at positions 296 and 310, which are known to be redoxsensitive (Supporting Information, Figure S1).[15] Cys296 and Cys310 are located in close proximity to these inhibitors and could be potentially addressed by cysteine reactive probes.

We therefore set out to combine the outstanding selectivity of PH domain-dependent Akt inhibitors[16] with pharmacological and therapeutic benefits of targeted irreversible modulators, which include superior drug-target residence time and increased potency.[5c] Herein we report our efforts to amalgamate these features and promote the design, synthesis,

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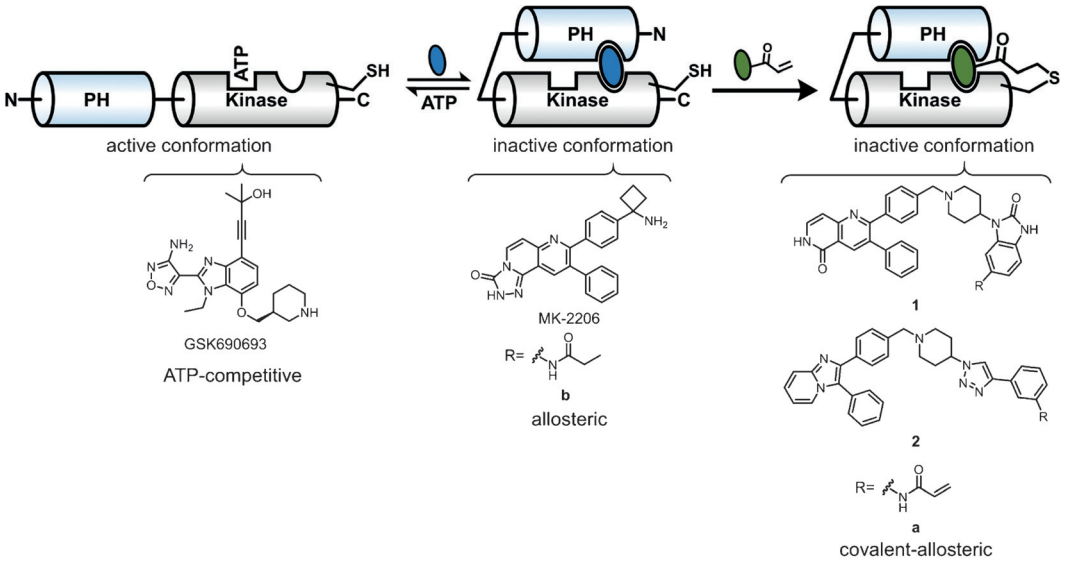

Figure 1. Representations of the structures of Akt in the active and inactive conformation. ATP-competitive inhibitor targeting the kinase domain of Akt (left). Allosteric PH domain-dependent Akt inhibitors (blue) bind reversibly to an interface formed by the kinase domain and the PH domain of Akt (middle). Covalent-allosteric PH domain-dependent inhibitors (green) irreversibly stabilize the enzymatically inactive 'PH-in' conformation. Structures of covalent-allosteric PH domain-dependent Akt inhibitors **1a** and **2a** and their reversible analogues **1b** and **2b**.

and biochemical and cellular characterization of PH domain-dependent covalent-allosteric inhibitors (CAIs) as a novel approach to target Akt and to probe its signaling and functions beyond catalysis.

Based on the previously described 1,6-naphthyridinone scaffold[17] and an imidazo-1,2-pyridine core,[18] we designed and synthesized chemically diverse probe compounds (**1a** and **2a**) featuring Michael acceptors to target Cys296 or Cys310 (Figure 1). Additionally, we synthesized **1b** and **2b** as reversible counterparts. All inhibitors were subjected to kinetic analyses using the interface fluorescent labels in kinases (iFLiK) system[19] that allows for the detection of PH domain-dependent binders for Akt to determine their dissociation rates with respect to fluorescently labeled full-length Akt1 (Table 1; Supporting Information, Figure S2).

These results indicated similar binding affinities for probe compound **1a** and reference inhibitor MK-2206 (58 ± 8 nM and 69 ± 13 nM, respectively), whereas **2a** exhibited a significantly lower binding affinity for Akt1 (795 ± 176 nM). The reversible counterparts **1b** and **2b**, however, possessed comparable K_d values (62 ± 12 nM and 797 ± 180 nM, respectively). These results were consistent with the fact that the full-length Akt protein used in these studies had been mutated for site-specific fluorescent labeling by replacing all solvent-exposed cysteines with serines, including both A-loop cysteines. We evaluated the potential influence of the Michael acceptor and further assessed the inhibitory potencies of our warhead-decorated probes in activity-based studies utilizing activated full-length wtAkt1 (Table 1; Supporting Information, Figure S3) as well as activated ΔPH-Akt1 (Supporting Information, Figure S4). Whilst probe molecule **2a** moderately inhibited Akt kinase activity in comparison to reference inhibitors MK-2206 and GSK690693 (372 ± 48 nM vs. 7 ± 1 nM and 2 ± 1 nM, respectively), probe compound **1a** exhibited a subnanomolar half-maximal inhibitory concentration (0.2 ± 0.1 nM). In contrast, tenfold and fourfold higher concentrations of **1b** and **2b**, respectively, were required to provoke half-maximum inhibition of Akt1, suggesting the covalent modification of Akt by **1a** and **2a** as reason for their higher potency. Additionally, time-dependent IC_{50} determinations of **1a** further indicated a covalent mode of action (Supporting Information, Figure S5). Kinetic characterization of our probe compounds by determination of k_{inact}/K_i indicated a significantly enhanced inhibition profile for **1a** compared to **2a**, with respect to both affinity and covalent complex formation (Table 1; Supporting Information, Figure S6). Moreover, superior inhibition properties were verified for our covalent probes when compared to the clinical lead compound MK-2206, which is currently being investigated for the treatment of HER2-positive solid tumors and acute myelogenous leukemia.[20]

Table 1: Half-maximal inhibitory concentrations (IC_{50}), k_{inact}/K_i, and dissociation constants (K_d) of reference compounds and CAIs.[a]

		wtAkt1	iFLiK-Akt1 (E49C/C296S/C310S/C344S)
Compound	IC_{50} [nM]	k_{inact}/K_i [$\mu M^{-1} s^{-1}$]	K_d [nM]
MK-2206	6.5 ± 0.8	–	69 ± 13
GSK690693	2.3 ± 0.3	–	n.r.
1a	0.2 ± 0.1	3.29 ± 0.40	58 ± 8
1b	7.5 ± 2	–	62 ± 12
2a	372 ± 48	0.0023 ± 0.0003	795 ± 176
2b	992 ± 328	–	797 ± 180

[a] n.r. = no response up to 100 μM.

After evaluating the inhibitory effects of the compounds, we utilized ESI-MS to investigate their respective binding properties to Akt1. Treatment of recombinant wild-type Akt1 with probe compounds **1a** and **2a** resulted in mass increases equivalent to the corresponding single labeled Akt1 (587 and

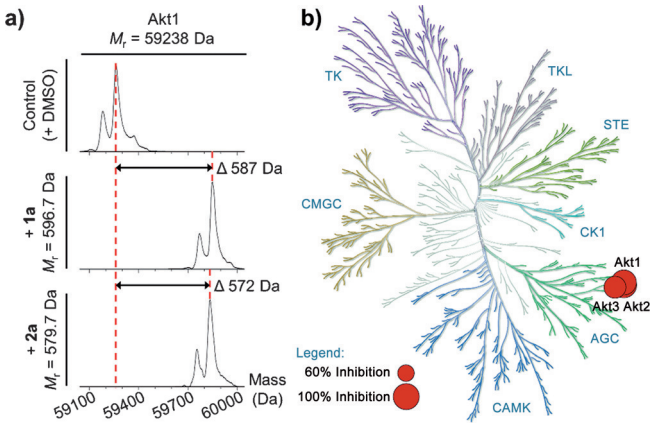

Figure 2. a) Deconvoluted mass spectra of Akt1 incubated with DMSO (top), **1a** (middle), and **2a** (bottom), respectively. Mass differences in relation to DMSO-treated control are displayed as Δ values and demonstrate covalent modification of Akt1 with **1a** and **2a**. b) Kinome dendrogram of **1a**. At 1 μM compound concentration, only Akt1, Akt2, and Akt3 were significantly inhibited (98%, 96%, and 83%) whereas the remaining 97 kinases were inhibited by less than 40%. Kinase profiling data were generated with the SelectScreen Profiling Service from LifeTechnologies. The kinase dendrogram was adapted from Kinome Render[22] and the illustration is reproduced with courtesy of Cell Signaling Technology, Inc. (http://www.cellsignal.com).

572 Da, respectively), as compared to control Akt1 treated with DMSO (Figure 2). These results additionally corroborated the biochemical data and further substantiated the anticipated covalent binding mode correlating with the observed trends in activity-based studies (Table 1).

After tryptic digest of the **1a**- and **2a**-treated Akt and ESI-MS/MS analysis, we identified Cys296 and Cys310 as the modification sites for our probes (Supporting Information, Figure S7). The respective mass signatures of both modified cysteine-containing peptide fragments accounted for Cys296_**1a**, as well as Cys310_**1a**. Analogous findings were obtained for probe **2a**. These results indicated that Cys296 was not preferentially targeted over Cys310 or vice versa by our probes and further substantiated our hypothesis that both cysteines located on the flexible activation loop are accessible for covalent modification by suitable electrophile-decorated inhibitors (Supporting Information, Figure S7).

We next investigated the selectivity of **1a** for Akt1 over other protein kinases with similar characteristics focusing on a) members of the AGC kinase family, b) kinases featuring a PH domain, and/or c) kinases containing cysteines on the activation loop. One hundred different protein kinases matching these criteria were assessed employing the SelectScreen Kinase Profiling Services from Life Technologies (Supporting Information, Table S1).

Within the panel of kinases, solely the Akt isoforms 1–3 were significantly addressed (>80% inhibition) at a concentration of 1 μM of **1a** (Supporting Information, Table S1). MAP4K5, which was previously shown to be moderately inhibited by a substrate-competitive phenylalanine-based Akt inhibitor,[21] ranked fourth with 37% inhibition. Interestingly, MAP4K5 contains neither an activation loop cysteine, nor a PH domain and it does not belong to the AGC superfamily. Furthermore, conserved cysteines analogous to Cys296 and Cys310 most likely do not account for off-target activity as deduced from the poor inhibition profile of PKCθ (35% inhibition), MAPKAPK2 (30%), and MELK (30%) as well as 43 additional kinases harboring analogous cysteines (<30% inhibition). Therefore, probe compound **1a** exhibited an excellent selectivity profile, exclusively targeting Akt isoforms without affecting kinases revealing high sequence and structural homology.

We further characterized our most promising CAI **1a** in cellular studies using prostate cancer (PC3), breast cancer (BT474), and gastrointestinal stromal tumor (GIST-T1) cell lines, which possess genetic lesions (PC3: PTEN-/-, BT474: PI3K mut, HER2+; GIST-T1: c-KIT mut) in the PI3K/Akt pathway and display differences in basal phospho-Akt levels. These cell lines were employed as model systems for Western Blot analyses to investigate both the cell penetrating effect of **1a** as well as its influence on cellular Akt1 and downstream GSK3β phosphorylation states. Treatment with MK-2206 (reference compound) and with **1a** induced sensitive dose-dependent decreases of pAkt1 at both Thr308 and Ser473 in PC3 and BT474 cancer cell lines; these results were correlated with decreased phosphorylation of the Akt1 substrate GSK3β (Supporting Information, Figure S8).

Moreover, using c-KIT-dependent GIST-T1 cells, we demonstrated the selectivity of **1a** for Akt1 in a cellular setting, whilst sparing further oncogenic protein kinases, such as c-KIT and Erk1/2 (Figure 3). Therefore, **1a** was demonstrated to be a cell-permeable effector of Akt in various cancer cell lines. These observations introduce the prospect of utilizing our probe compounds for further medicinal chemis-

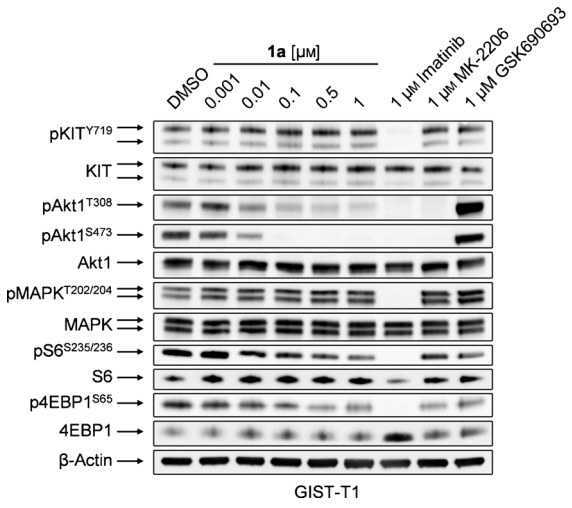

Figure 3. Covalent-allosteric inhibitor **1a** impairs Akt1 phosphorylation in cancer cells. Gastrointestinal stromal tumor cells (GIST-T1) exhibit high basal pAkt1 levels related to constitutively active c-KIT (imatinib: positive control for c-KIT inhibition). Treatment with CAI **1a** reduces cellular pAkt1 levels at low nanomolar concentrations correlating with decreasing phosphorylation of downstream targets S6 and 4EBP1; phosphorylation of other relevant protein kinases c-KIT and MAPK (Erk1/2) is not affected by treatment with **1a**. Two bands are visible for c-KIT representing the mature glycosylated form and the immature form. Regarding MAPK, the bands relate to Erk1 and Erk2.

try approaches. Such new compounds may contribute to the development of covalently modulating anticancer drugs.

In summary, we rationally designed, synthesized, and characterized novel allosteric inhibitors that covalently modify Akt at Cys296 and Cys310. Single labeling of the protein with our covalently modulating inhibitors at the desired cysteines was confirmed by mass spectrometry. Further to promising biochemical inhibitory potency against Akt and outstanding selectivity over other protein kinases with homologous structure and similar sequence, **1a** was demonstrated to exhibit analogue impact as compared to the current clinical lead candidate MK-2206 in cellular model systems. Moreover, we demonstrated that targeting non-catalytic cysteines in Akt is feasible by chemically diverse methods that can likewise be used as functional probes for Akt modulation in chemical and systems biology endeavors. This proof-of-principle study illustrates the tremendous potential of CAIs in the targeted therapy of patients suffering from tumors with aberrantly activated Akt and, above all, underlines the importance of defining and understanding fundamental molecular mechanisms that induce, regulate, and control cancer development.

Keywords: cancer · drug design · inter-domain interactions · medicinal chemistry · tumor thermapeutics

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